

See discussions, stats, and author profiles for this publication at: <https://www.researchgate.net/publication/6821971>

Short report: ITS-1 DNA sequence confirmation of *Leishmania major* as a cause of cutaneous leishmaniasis from an outbreak focus in the Ho District, southeastern Ghana

Article in *The American journal of tropical medicine and hygiene* · October 2006

DOI: 10.4269/ajtmh.2006.75.502 · Source: PubMed

CITATIONS

11

READS

67

13 authors, including:



David J Fryauff

Naval Health Research Center

81 PUBLICATIONS 2,967 CITATIONS

[SEE PROFILE](#)



John D Klena

Centers for Disease Control and Prevention

361 PUBLICATIONS 4,759 CITATIONS

[SEE PROFILE](#)



David F Hoel

Uniformed Services University of the Health Sciences

62 PUBLICATIONS 451 CITATIONS

[SEE PROFILE](#)



Naiki Pupilampu

Noguchi Memorial Institute for Medical Research

20 PUBLICATIONS 117 CITATIONS

[SEE PROFILE](#)

Some of the authors of this publication are also working on these related projects:



Establishing Reference Value for Kintampo North and South, for the Phase III trial of the RTS,S malaria vaccine [View project](#)



Surveillance of antimalarial resistance in Ghana [View project](#)

SHORT REPORT: ITS-1 DNA SEQUENCE CONFIRMATION OF *LEISHMANIA MAJOR* AS A CAUSE OF CUTANEOUS LEISHMANIASIS FROM AN OUTBREAK FOCUS IN THE HO DISTRICT, SOUTHEASTERN GHANA

DAVID J. FRYAUFF, HANAFI A. HANAFI, JOHN D. KLENA, DAVID F. HOEL,* MAXWELL APPAWU, WILLIAM ROGERS, NAIKI PUPLAMPU, SHIRLEY ODOOM, MARGARET KWEKU, KWADWO KORAM, MICHAEL D. WILSON, GREGORY RACZNIAK, AND DANIEL BOAKYE

Research Science Directorate; Enteric Disease Research Program; Vector Biology Research Program; Ghana Detachment, U.S. Naval Medical Research Unit 3, Cairo, Egypt; Infectious Disease Directorate, Naval Medical Research Unit, Silver Springs, Maryland, USA; Department of Parasitology; Department of Epidemiology; Department of Entomology; Noguchi Memorial Institute for Medical Research, Accra, Ghana; Ho District Directorate, Ghana Health Service, Ho, Ghana

We report first-time definitive evidence of human cutaneous leishmaniasis (CL) resulting from *L. major* infections in Ghana. The evidence is in the form of PCR testing and DNA sequence analysis of skin sample biopsies from residents of the Ho District in the forested Volta Region of southeastern Ghana (Figure 1). The moist semi-deciduous forest of this region of Ghana is atypical for CL. Although two sand fly species with known or suspected potential to transmit *L. major* were present in communities where human cases of CL originated, they were found in such small numbers that their role in the transmission of CL is uncertain.

Twelve million people worldwide are believed to be infected with the parasites that cause leishmaniasis, and 88 different nations report this disease.¹ It is estimated that up to 75% of the 1.5 million new cases of leishmaniasis that occur each year are CL with *L. major* the responsible etiologic agent of this cutaneous disease in Africa and throughout Eurasia. Over this vast range, CL transmission is typically associated with arid desert and savannah conditions where the parasites are harbored in rodents and vectored by night-biting sand flies of the genus *Phlebotomus*.

For many years, leishmaniasis in Africa and Western Asia has been grossly underestimated; it now appears that the disease is much more prevalent than previously suspected.¹ In Ghana, CL was considered a parasitic disease risk that was limited to the arid sahel-savannah region in the north, but no infections or evidence of transmission had been actually described. Thousands of man-hours of mosquito surveillance have been conducted in northern Ghana² for the purpose of quantifying man-biting malaria vectors, but there is no record or report of sand flies in these collections. However, since 1999, an increasing number of suspected CL cases have been reported from the southern Volta Region of Ghana in a moist forest ecosystem where such skin lesions had not been previously reported. The assumption of CL was based subjectively on local microscopy, which identified *Leishmania* amastigotes in skin lesion biopsies taken from a cluster of local patients. Between 1999 and 2002, the Ghana Health Service recorded 2,426 suspected cases of CL in the Ho, Hohoe, and Kpando Districts (2,348, 2, and 76 cases, respectively). In 2003, the number of suspected cases rose to 6,450 (6,185, 174, and 91 in the same respective districts) with 116 villages affected (Ghana Ministry of Health (MoH), Annual Report, 2003). A

limited survey of towns in the Ho district during 2002 identified suspected CL lesions in 12.2–32.3% of local school children. In an effort to determine the cause and extent of the outbreak, the Ghana MoH initiated an epidemiologic study in Ho district with assistance from the U.S. Naval Medical Research Unit No. 3 and the Noguchi Memorial Institute of Medical Research.

Initial efforts of our study intended to verify the presence of *Leishmania* parasites by applying newly developed PCR methods to 5 previously collected, archived biopsy samples embedded in paraffin and if possible, to identify the species of parasite and its genetic relatedness to a variety of reference strains of *Leishmania*. Entomological surveillance based on sand flies collected from oiled paper trapping and CO₂-baited miniature CDC light traps conducted from May 2004–February 2005 sought to identify candidate vectors and their population trends in the affected region. Subsequently, the Ghana Health Service requested PCR-based diagnoses on newly collected needle biopsies from patients seeking diagnosis and treatment of skin lesions at local health clinics and the district hospital in Ho. Diagnostic reagents were provided and technology was transferred from NAMRU-3 to the Noguchi Memorial Institute for Biomedical Research to establish methods for PCR diagnosis of Leishmaniasis in Ghana by Ghanaian investigators and healthcare workers.

Verbal informed consent was obtained from villagers with suspected leishmaniasis. The lesion site was cleaned with Betadine solution. Using a 3-cc syringe, the biopsy area was anesthetized with 1% lidocaine and draped in a sterile fashion. A ~0.5 × 0.25 cm section of the lesion was excised with a #15 scalpel from subcutaneous fat. The elliptical section was removed and fixed in 100% ethanol for storage and transport. Adequate homeostasis was observed and a sterile dressing was applied. DNA was later extracted from paraffin-embedded tissue by first making thin slices of the paraffin and subsequently removing as much of the paraffin with a scalpel as possible. Remaining sample was sliced into small pieces and no more than 25 mg of tissue was mixed with 1 mL of xylene and extracted by a standard method.³ After paraffin-removal, DNA was extracted from the tissue using the Qiagen DNA mini kit (Qiagen, Valencia, CA) as per the manufacturer's recommendations. DNA was resuspended in 100 μL of AE buffer and stored at 4°C until use.

A foot lesion from a BALB/c mouse experimentally infected with *Leishmania major* IPAP/EG/89/SI-177⁴ was used as a positive control for genetic manipulations. The DNA was extracted from 25 mg of the mouse tissue using the Qiagen DNA mini kit. To avoid cross-contamination, the BALB/c

* Address correspondence to David F. Hoel, Vector Biology Research Program, U.S. Naval Medical Research Unit No. 3, PSC 452 Box 141, FPO AE 09835. E-mail: hoeld@namru3.med.navy.mil

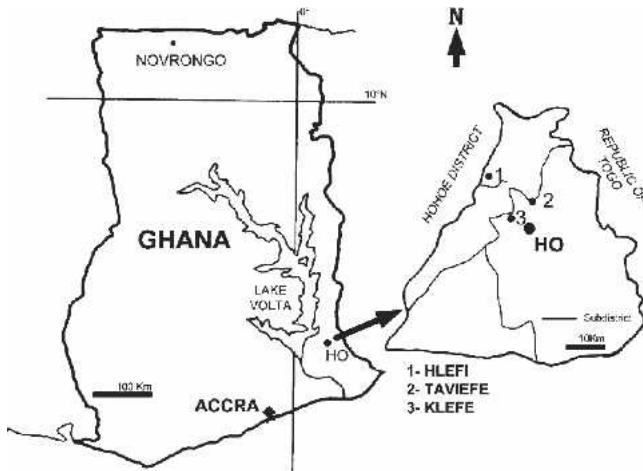


FIGURE 1. Map of Ghana showing the location of Ho district and outlet showing the areas where human cases of leishmaniasis occurred and where sand flies were collected.

mouse tissue was extracted in a separate room from the paraffin slices, on separate occasions, and a clean (non-infected) control was simultaneously prepared.

Target DNA was amplified from extracted DNA using previously described primers and conditions.⁵⁻⁷ Primers R174 (5'-ggg tcc ttt cct gat tta cg) and R798 (5'-ggc cgg taa agg ccg aat ag)⁶ were used to amplify a 603-bp product and primers LITSR (5' ctg gat cat ttt ccg atg) and L5.8S (5' tga tac cac tta tcg cac tt)⁵ were used to amplify a 336-bp fragment from the DNA extracted from paraffin-embedded tissue and control DNA. Six μ l of paraffin-extracted and 2 μ L of control BALB/c DNA, respectively, were used as a template for each PCR reaction. A reaction containing no DNA was used as a negative control for each assay. Amplicons were visualized after electrophoresis through 1% agarose containing a final concentration of 10 μ g ml⁻¹ ethidium bromide. Images were captured using a Kodak EDAS 290 image capture system.

PCR products were processed using the Qiagen PCR clean-up kit (Qiagen, Valencia, CA) to remove excess oligonucleotide primer and to concentrate the product. PCR products were subsequently directly sequenced using Big Dye Terminator Technology (ABI Biosystems, Foster City, CA) and primers R174, R798, LITSR, and L5.8S at 3.2 pmol per reaction, using a 3100 Avant Genetic Analyzer (ABI Biosystems, Foster City, CA). DNA sequences were manipulated and analyzed using the software program pDRAW32 1.1.87 (<http://www.acaclone.com>) and aligned using the program Clustal X.⁸ Amended sequences were sent to the BLAST nucleotide data base (BLAST 2.2.12) for interrogation. DNA accession numbers are as follows: ITS-1 DNA sequences from *L. major* IPAP/EG/89/SI-177, DQ295824; MHOM/GH/04/HO-004, DQ295825; ssu rDNA from *L. major*, IPAP/EG/89/SI-177, DQ295827 and from MHOM/GH/04/HO-004, DQ295826. *Leishmania major* ITS1 sequences from 3 Iranian isolates (MHOM/Ir/02/PIICC1, MHOM/Ir/02/PIIDT1, and MHOM/Ir/00/PII22; accession numbers AY260965, AY283793, and AY573188), a Kenyan isolate (MTAT/KE/??/NLB089A; accession number AJ300482), a Sudanese isolate (MHOM/SD/90/SUDAN3; accession number AJ300481), a Russian isolate (MHOM/SU/73/5ASKH; accession number AJ000310), and an isolate from Turkmenistan (Vin; accession

number AJ272383) were used for comparison. For purposes of DNA comparison, ITS-1 sequences from additional Old World *Leishmania* species were also included. These include 3 isolates of *L. donovani* (MHOM/ET/00/Hussen, AJ634360; MHOM/KE/83/NLB189, AJ634374; and MHOM/SD/68/1S, AJ000293), 2 isolates each of *L. archibaldi* (MHOM/ET/72/GEBRE1, AJ634367 and MHOM/SD/97/LEM3429; AJ634358), and *L. infantum* (MOHM/SD/62/3S, AJ634361 and MHOM/TN/80/IPT1, AJ000289). A single isolate of *L. tropica* (MHOM/TN/88/TAT3, AJ300485) was also used. Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 3.1.⁹ A phylogenetic tree (Figure 2) was constructed with the unweighted pair group method with arithmetic mean analysis.¹⁰ Evolutionary distances were calculated with the method of Jukes and Cantor.¹¹ Bootstrap analysis¹² was performed with 100 replicates.

DNA extraction and PCR demonstrated that the paraffin-embedded tissue samples from Ghanaian patients with skin lesions were positive for *Leishmania* species using both conventional primer sets ssu-rDNA and ITS1. The ITS1 PCR product from MHOM/GH/04/HO-004 was direct sequenced and shown to contain a HaeIII restriction site at position 132 bp. The ITS1 product would subsequently resolve into two fragments of 132 and 204 bp, consistent with the sizes shown for the ITS1 product of *L. major*. Interrogation of the BLAST nucleotide database revealed that the *Leishmania* species DNA from the Ghana paraffin-embedded tissues shared almost complete identity with the ITS1 sequences of *L. major*, specifically with an African isolate of *L. major* from Kenya.

Over 2,200 sand flies were collected during 10 consecutive

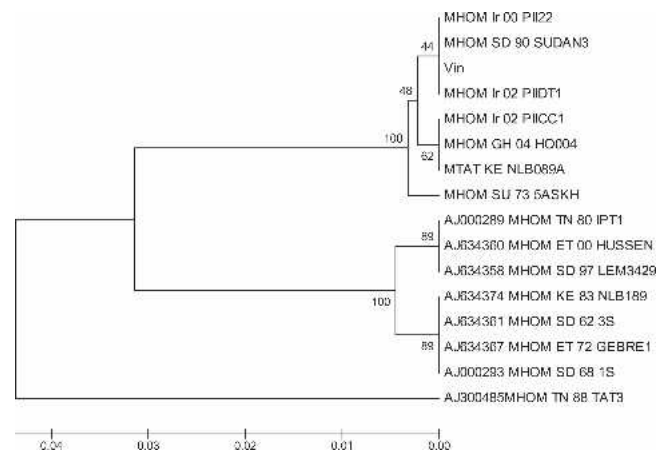


FIGURE 2. Dendrogram based on *L. major* ITS1 sequence comparison. 336 bp of the ITS1-5.8S ribosomal RNA region from representative *L. major* isolates were aligned and a dendrogram was constructed using the unweighted pair group method with arithmetic mean analysis with evolutionary distance calculated using the method of Jukes and Cantor. Bootstrap analysis was performed with 100 replicates. Strain accession numbers were: IPAP/EG/89/SI-177 (this study), DQ295824; MHOM/GH/04/HO-004 (this study), DQ295825; MHOM/Ir/02/PIICC1, AY260965; MHOM/Ir/02/PIIDT1, AY283793; MHOM/Ir/00/PII22, AY573188, MTAT/KE/NLB089A, accession AJ300482, MHOM/SD/90/SUDAN3, AJ300481; MHOM/SU/73/5ASKH, AJ000310; and Vin, AJ272383. Accession numbers for other Old World *Leishmania* species are *L. donovani* MHOM/ET/00/Hussen, AJ634360; MHOM/KE/83/NLB189, AJ634374; and MHOM/SD/68/1S, AJ000293; *L. archibaldi*, MHOM/ET/72/GEBRE1, AJ634367 and MHOM/SD/97/LEM3429; AJ634358; *L. infantum*, MOHM/SD/62/3S, AJ634361 and MHOM/TN/80/IPT1, AJ000289; and *L. tropica* MHOH/TN/88/TAT3, AJ300485.

TABLE 1
Species composition and abundance of Phlebotomine sand flies collected from Ho District, Ghana

Species	Ho	Percent capture
<i>S. africana</i> *	812	35.6
<i>S. squamipleuris</i> *	801	35.1
<i>S. ingrami</i>	347	15.2
<i>S. simillima</i>	119	5.2
<i>S. schwetzi</i>	42	1.8
<i>S. dubia</i>	41	1.8
<i>S. durenii</i>	25	1.1
<i>S. antennata</i>	24	1.1
<i>S. hamoni</i>	20	0.9
<i>S. buxtoni</i>	14	0.6
<i>S. collarti</i>	11	0.5
<i>S. dissimillima</i> *	6	0.26
<i>P. rodhaini</i> *	6	0.26
<i>S. affinis</i>	4	0.2
<i>S. clydei</i>	4	0.2
<i>P. duboscqi</i> *†	3	0.13
<i>S. pastoriana</i>	1	0.04
Total	2280	

* Newly recorded in Ghana; † Known leishmaniasis vector.
P., *Phlebotomus*; *S.*, *Sergentomyia*.

months of surveillance in the Ho District (May 2004–February 2005). Sand fly identifications were made using the taxonomic keys of Abonnenc¹³ and Niang et al.¹⁴ Microscopic dissection and examination of 722 individual female sand flies captured in light traps revealed no *Leishmania* infections. *Phlebotomus duboscqi*, a proven vector of *L. major* in Senegal, Burkina Faso, Nigeria, and Kenya had been previously reported from Ghana¹⁵ and was considered our primary suspected vector of CL in the Ho District. Surprisingly, however, *P. duboscqi* was near least abundant of 17 different sand fly species (including 6 new geographic records for sand flies in Ghana) that we collected and comprised only 0.1% of the total catch (Table 1). One other suspect sand fly species, *P. rodhaini*, was captured for the first time in Ghana, but at similarly low numbers (0.3%), insufficient to yield a profile of seasonal abundance. In Sudan, *P. rodhaini* was found to harbor an unknown *Leishmania* species and demonstrated a clear predilection for blood feeding on rodents.¹⁶

This is the first confirmation of *L. major* in Ghana. Our finding might have been expected in the arid sahel-savannah regions of northern Ghana bordering the West African *L. major* belt; however, the current outbreak was unexpected in its intensity and occurrence in a moist semi-deciduous forest ecosystem. This finding was especially surprising in view of the intensive entomological sampling that was conducted and results that give rise only to more questions. Significantly, we established a proven PCR-based methodology for detection and identification of *Leishmania* in Ghana for use by Ghanaian scientists and healthcare professionals. Ongoing epidemiologic surveillance efforts will hopefully elucidate vectors, reservoirs, and contributing factors responsible for the large occurrence and seemingly progressive spread of *L. major* in an area of Ghana where it was not known previously to have occurred.

Received January 26, 2006. Accepted for publication March 31, 2006.

Authors' addresses: David J. Fryauff, U.S. Naval Medical Research Center, 503 Robert Grant Avenue, Silver Spring, MD 20910. Hanafi A. Hanafi, John D. Klana, and David F. Hoel, U.S. Naval Medical

Research Unit No. 3, PSC 452 Box 141, FPO AE 09835. Maxwell Appawu, Naiki Puplampu, Kwadwo Koram, Michael D. Wilson, and Daniel Boakye, Noguchi Memorial Institute for Medical Research, P.O. Box LG 581, Legon Accra, Ghana. William O. Rogers, U.S. Naval Medical Research Unit No. 2, Box 3 Unit 8132, FPO AP 96520-8132. Shirley Odoom and Margaret Kweku, Ghana Health Service, Private Mail Bag, Ministries, Accra, Ghana. Gregory Racznik, Ghana Detachment, U.S. Naval Medical Research Unit No. 3, C/O Department of State, 2020 Accra Place, Washington, DC 20158-2020.

Reprint requests: David F. Hoel, Vector Biology Research Program, U.S. Naval Medical Research Unit No. 3, PSC 452 Box 141, FPO AE 09835. E-mail: hoeld@namru3.med.navy.mil

REFERENCES

- WHO, 2000. The Leishmaniases and Leishmania/HIV co-infections. *Fact Sheet No. 116*.
- Appawu M, Owusu-Agyei S, Dadzie S, Asoala V, Anto F, Koram K, Rogers W, Nkrumah F, Hoffman SL, Fryauff DJ, 2003. Malaria transmission dynamics at a site in northern Ghana proposed for testing malaria vaccines. *Trop Med Int Health* 9: 164–170.
- Cao W, Hashibe M, Rao JY, Morgenstern H, Zhang ZF, 2003. Comparison of methods for DNA extraction from paraffin-embedded tissues and buccal cells. *Cancer Detect Prev* 27: 397–404.
- Mansour NS, Fryauff DJ, Modi GB, Mikhail EM, Youssef FG, 1991. Isolation and characterization of *Leishmania major* from *Phlebotomus papatasi* and military personnel in north Sinai, Egypt. *Trans R Soc Trop Med Hyg* 85: 590–591.
- El Tai NO, Osman OF, El Fari M, Presber W, Schönian G, 2000. Genetic heterogeneity of ribosomal internal transcribed spacer in clinical samples of *Leishmania donovani* spotted on filter paper as revealed by single-strand conformation polymorphisms and sequencing. *Trans R Soc Trop Med Hyg* 94: 575–579.
- Meredith SE, Zijlstra EE, Schoone GJ, Kroon CC, van Eys GJ, Schaeffer KU, el-Hassan AM, Lawyer PG, 1993. Development and application of the polymerase chain reaction for the detection and identification of *Leishmania* parasites in clinical material. *Arch Inst Pasteur Tunis* 70: 419–431.
- Schönian G, Nasereddin A, Dinse N, Carola S, Schallig HD, Presber W, Jaffe CL, 2003. PCR diagnosis and characterization of *Leishmania* in local and imported clinical samples. *Diagn Microbiol Infect Dis* 47: 349–358.
- Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG, 1997. The Clustal X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res* 25: 4876–4882.
- Kumar S, Tamura K, Nei M, 2004. MEGA3: integrated software for Molecular Evolutionary Genetics Analysis and sequence alignment. *Brief Bioinform* 5: 150–163.
- Sneath PHA, Sokal RR, 1973. *Numerical Taxonomy*. San Francisco: W.H. Freeman and Company, 230–234.
- Jukes TH, Cantor CR, 1969. Evolution of protein molecules. Munro HN, ed. *Mammalian Protein Metabolism*. New York: Academic Press, 21–132.
- Felsenstein J, 1985. Confidence limits on phylogenies: an approach using the bootstrap. *Evolution Int J Org Evolution* 39: 783–791.
- Abonnenc E, 1972. *Les Phlebotomes de la Region Ethiopienne (Diptera, Psychodidae)*. Paris, ORSTOM.
- Niang AA, Geoffroy B, Angel G, Trouillet J, Killick-Kendrick R, Hervey JP, Brunches J, 2000. *Les phlebotomes d'Afrique de l'Quest*: Institut de recherche pour le développement, Institut fondamental d'Afrique noir l'Université Cheikh Anta Diop de Dakar.
- Beach R, Young DG, Kiilu G, 1986. New Phlebotomine sand-fly colonies. II. Laboratory colonization of *Phlebotomus duboscqi* (Diptera, Psychodidae). *J Med Entomol* 23: 114–115.
- Hoogstraal H, Deitlein DR, Heyneman D, 1962. Leishmaniasis in the Sudan Republic 4. Preliminary observations on man-biting sand flies (Psychodidae: *Phlebotomus*) in certain Upper Nile endemic areas. *Trans R Soc Trop Med Hyg* 56: 411–422.